THIS PAGE BLANK (USPTO)

ELSEVIER

Biochimica et Biophysica Acta 1490 (2000) 311–323

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.elsevier.com/locate/bba

Cloning and characterization of additional members of the G protein-coupled receptor family

Dennis K. Lee [a], Kevin R. Lynch [e], Tuan Nguyen [c], Dong-Soon Im [e], Regina Cheng [c], Victor R. Saldivia [a], Yang Liu [a], Ivy S.C. Liu [a], Henry H.Q. Heng [d], Philip Seeman [a], Susan R. George [a,b,c], Brian F. O'Dowd [a,c,*], Adriano Marchese [a]

[a] *Department of Pharmacology, University of Toronto, Medical Sciences Building, Toronto, Ont. M5S 1A8, Canada*
[b] *Department of Medicine, University of Toronto, Medical Sciences Building, Toronto, Ont. M5S 1A8, Canada*
[c] *Centre for Addiction and Mental Health, 33 Russell Street, Toronto, Ont. M5S 2S1, Canada*
[d] *See DNA Biotech, Inc., Farquharson Building, 4700 Keele Street, Downsview, Ont. M3J 1P3, Canada*
[e] *Department of Pharmacology, University of Virginia Health Sciences Center, Charlottesville, VA 22908, USA*

Received 31 August 1999; received in revised form 1 December 1999; accepted 7 December 1999

Abstract

A search of the expressed sequence tag (EST) database retrieved a human cDNA sequence which partially encoded a novel G protein-coupled receptor (GPCR) GPR26. A human genomic DNA fragment encoding a partial open reading frame (ORF) and a rat cDNA encoding the full length ORF of GPR26 were obtained by library screening. The rat GPR26 cDNA encoded a protein of 317 amino acids, most similar (albeit distantly related) to the serotonin 5-HT_{5A} and gastrin releasing hormone BB2 receptors. GPR26 mRNA expression analysis revealed signals in the striatum, pons, cerebellum and cortex. HEK293 and Rh7777 cells transfected with GPR26 cDNA displayed high basal cAMP levels, slow growth rate of clonal populations and derangements of normal cell shape. We also used a sequence reported only in the patent literature encoding GPR57 (a.k.a. HNHCI32) to PCR amplify a DNA fragment which was used to screen a human genomic library. This resulted in the cloning of a genomic fragment containing a pseudogene, ψGPR57, with a 99.6% nucleotide identity to GPR57. Based on shared sequence identities, the receptor encoded by GPR57 was predicted to belong to a novel subfamily of GPCRs together with GPR58 (a.k.a. phBL5, reported only in the patent literature), putative neurotransmitter receptor (PNR) and a 5-HT_4 pseudogene. Analysis of this subfamily revealed greatest identities (~56%) between the receptors encoded by GPR57 and GPR58, each with shared identities of ~40% with PNR. Furthermore, ψGPR57, GPR58, PNR and the 5-HT_4 pseudogene were mapped in a cluster localized to chromosome 6q22-24. PNR and GPR58 were expressed in COS cells, however no specific binding was observed for various serotonin receptor-specific ligands.

Keywords: Orphan G protein-coupled receptor; Pseudogene; Chromosome

1. Introduction

Over the past decade, cloning experiments have succeeded in identifying many GPCRs for which endogenous ligands are known. The search for novel

* Corresponding author. Fax: +1-416-971-2868; E-mail: brian.odowd@utoronto.ca

0167-4781/00/$ – see front matter © 2000 Elsevier Science B.V. All rights reserved.
PII: S0167-4781(99)00241-9

Applicants: Beth E. Borowsky, et al.
U.S. Serial No.: 09/980,145
Filing Date: November 28, 2001
Exhibit 7

GPCR genes has also identified a large cohort of genes whose products are members of the GPCR family but for which the ligands are not known [1]. The existence of these orphan G protein-coupled receptors (oGPCRs) demonstrates that many neurotransmitter-receptor systems remain to be identified and functionally characterized. Thus far, five endogenous ligands (nociceptin/orphanin FQ [2,3], orexin-A and B/hypocretin-1 and 2 [4], prolactin-releasing peptide [5] and apelin [6]) have been discovered by expression of several oGPCRs by the process of reverse pharmacology [7,8]. In each case, stable cell lines were transfected with DNA encoding the oGPCR, treated with tissue extracts and assayed for signal transduction. Thus, oGPCRs can be used to analyze anatomic expression and ligand identification. We have continued our search for novel GPCRs utilizing conserved sequences as molecular probes of the expressed sequence tag (EST) databases. The results from these searches are scanned against a database of ESTs, and the novel clones identified are obtained from the I.M.A.G.E. Consortium [9].

We now report the characterization of an oGPCR named GPR26. From a patent (#EP 0859055-A/1) we obtained the sequence HNHCI32 encoding an oGPCR (now renamed GPR57). Using this sequence we obtained a related pseudogene ψGPR57. Also from a patent (#JP 1997051795-A/1) we obtained the sequence phBL5, an oGPCR (renamed GPR58). We have attempted ligand binding of GPR26, GPR58 and the related oGPCR named PNR [10]. We present the chromosomal localization of GPR26, ψGPR57 and GPR58. Based on their chromosomal localization and identity to each other, genes GPR57, ψGPR57, GPR58, PNR and a 5-HT_4 pseudogene [11] appear to comprise a novel biogenic amine-like receptor subfamily of GPCRs.

2. Materials and methods

2.1. Materials

HEK293 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). 1321N1 cells were a gift from Dr. Myron Toews, Department of Pharmacology, University of Nebraska, NE, USA. Rh7777 cells were a gift from Dr. Jeorld Chun, Department of Pharmacology, University of California at San Diego, CA, USA. Pre-mixed cell culture media and media supplements, including serum and geneticin, were purchased from Life Technologies. EST cDNAs were from the ATCC. DNA sequence was determined by the University of Virginia's Biomedical Research Core Facility.

2.2. Cell culture

Human embryonic kidney 293, rat hepatoma Rh7777, human astrocytoma 1321N1 and COS-7 cells were grown at 37°C in a humidified atmosphere of 5% CO_2/95% air. HEK293 cells were cultured in DMEM/F12 supplemented with fetal bovine serum (10%). 1321N1 cells were cultured in DMEM supplemented with fetal bovine serum (5%). Rh7777 and COS-7 cells were grown in MEM supplemented with fetal bovine serum (10%).

2.3. DNA-mediated transfection

HEK293, 1321N1 and Rh7777 cells were transfected by a standard $CaPO_4$ DNA precipitate technique [12] and stably transfected cell populations were selected by growing cultures in medium supplemented with 800 μg/ml geneticin. COS-7 cells were transfected with a calcium phosphate transfection system (Gibco BRL, Gaithersburg, MD, USA).

2.4. oGPCR gene and cDNA cloning

A human EST cDNA clone (cloneID_HIBB05) partially encoding a novel human GPCR was used to screen a genomic DNA library (λEMBL Sp6T7, Clontech, Palo Alto, CA, USA) as previously described [13]. Oligonucleotide primers designed to amplify a single exon region of the EST cDNA were used to amplify a fragment of the rat orthologous gene. The rat DNA fragment was used to screen a rat brain cDNA library (5′ stretch, Clontech), resulting in the isolation two phage clones with cDNA inserts of 5.5 and 2.0 kb. Both cDNAs had identical translational ORFs encoding GPR26.

Human genomic DNA was amplified by PCR using oligonucleotide primers based upon sequences

encoding GPR57 (GPR57-1: 5′-CTCATCCTCCTG-GAAAGA-3′; GPR57-2: 5′-TAACAATCTCATTT-GCAA-3′), GPR58 (GPR58-1: 5′-TGCTCAGT-GBCDATEGAFHG-3′, (B = G or T; D = A, C, G, or T; E = A, C, or T; F = C or T; H = A or C); GPR58-2: 5′-ACCATATATTAACGGATT-3′) and PNR (PNR1: 5′-ATGAGAGCTGTCTTCATC-3′; PNR2: 5′-TCATTCTTGGTACAAATC-3′). PCR conditions were as follows: 94°C for 40 s, 50°C for 40 s, and 72°C for 1 min, for 35 cycles, followed by a 7 min extension at 72°C. PCR product bands in the expected size range were subcloned into the *Eco*RV site of pBluescript SK(−) or pcDNA3 (Stratagene) and sequenced. Inserts encoding ψGPR57 and GPR58 were used to screen a human genomic library (λEMBL Sp6T7, Clontech) as previously described [13]. ψGPR57 positive phage clones were plaque purified, amplified with GPR57 specific primers and sequenced. GPR58 positive phage clones were plaque purified, digested with several endonucleases, and subjected to Southern blot analysis as previously described [13].

To construct the full-length ORF of GPR58, two DNA fragments encoding the two exons of GPR58 were amplified and joined from human genomic DNA by PCR. Fragment 1 was amplified using oligonucleotide primers based upon the GPR58 5′ untranslated region (UTR) (GPR58-3: 5′-TGA-CAAAATTCTATCTGTTCTTG-3′) and the 3′ end of exon 1 (GPR58-4: 5′-CATACTATATGGCAT-GATGG-3′). Fragment 2 was amplified using primers based upon the 5′ end of exon 2 (GPR58-5: 5′-ATCAGATCGGTGGAGAACTGC-3′) and the sequence surrounding the stop codon (GPR58-6: 5′-TGCAGAAAAAGCCTACTCACTTTC-3′). PCR conditions were as follows: 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, for 35 cycles, followed by a 7 min extension at 72°C. The PCR products were subcloned into pCR 2.1-TOPO with the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA) and sequenced. Fragments 1 and 2 were joined and amplified to form the full GPR58 ORF by two further rounds of PCR. Round one consisted of fragments 1 and 2 undergoing 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min using primers whose sequence spanned both fragments (GPR58-7: 5′-ATAGTATGATCAGATCGGTGG-AGA-3′; GPR- 58-8: 5′-GATCTGATCATACTA-TATGGCATG-3′). A second round of PCR amplified an aliquot of the first round with primers GPR58-3 and GPR58-6 for 25 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 1 min. The PCR products were subcloned into pcDNA3 and sequenced to verify correct orientation for expression.

2.5. *cAMP accumulation*

Cells were plated in 12 well culture dishes and when the populations reached about 80% of confluency, growth medium was removed, cell monolayers rinsed with sterile buffered salts solution and overlaid with HKRB buffer (in mM: HEPES 20, NaCl 103, KCl 4.8, $CaCl_2$ 0.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 15, pH 7.4) supplemented with 0.1% fatty acid free bovine serum albumin (FAF-BSA). Cultures were treated with isobutylmethylxanthine (1 mM) for 15 min at 37°C. The assay was terminated by the addition of 0.5 ml of 0.15 N HCl. Following centrifugation to remove cell debris, the cAMP in the supernatant fluid was measured in an automated immunoassay (GammaFlow).

2.6. *Oocytes*

Oocytes were removed from anesthetized, adult *Xenopus laevis* toads through a small incision in the ventral wall. Stage V and VI oocytes were isolated manually from the ovary and incubated in collagenase (Type 1A, 1 mg/ml) in calcium-containing buffer for 2 h at room temperature. The absence of adherent material, including follicular cells, was confirmed by microscopic examination. Chloride currents were measured in response to applied compounds from oocytes held under two electrode voltage clamp as described previously [14].

2.7. *Calcium measurements*

These determinations were made as described previously [14,15]. Briefly, 1321N1 cells were trypsinized, collected by centrifugation, washed with HKRB buffer supplemented with 0.1% FAF-BSA. The cells were suspended in HKRB containing 10 μg/ml INDO-1/AM and after 45 min, washed, resuspended (at about 1.5×10^6 cells/ml) in HKRB without INDO-1 and kept for no more than an addi-

tional 90 min. Records of intracellular calcium fluxes were made on populations of $1-2\times10^6$ cells/ml in 2 ml cuvettes in a temperature-controlled fluorimeter (Aminco SLM 8000; SLM Instruments, Urbana, IL, USA). The cuvette was illuminated with light at 332 nm (slit width 4–8 nm) and emitted light was recorded at 400 and 485 nm (slit widths 4–8 nm). Calcium measurements were peak responses in traces of the ratio of the emitted light. Maximum and minimum fluorescence ratios were calculated by the sequential addition of digitonin (to 75 μM) and EDTA (to 5 μM). After testing lipid mediators, cultures were treated with 100 μM thrombin receptor agonist peptide (SFLLRNamide) to ascertain that the signaling pathway remained intact.

2.8. Radioligand binding assays

Membranes of COS-7 cells transfected with PNR were harvested and ligand binding assays performed as previously described [16] using [^{3}H]spiperone, [^{3}H]LSD, [^{3}H]5-HT, [^{3}H]5CT, [^{3}H]8-OH-DPAT, [^{3}H]Ketanserin and [^{3}H]GR113808 (Amersham, Uppsala, Sweden) as ligands. Membranes of COS-7 cells transfected with GPR58 were harvested and ligand binding assays were performed as previously described [17] using [^{3}H]5-HT against competitive displacement with methiothepin, mianserin and SDZ-205,557 HCl (RBI, Natick, MA, USA).

2.9. Northern blots

RNA from several human and rat brain regions and peripheral tissues was extracted by the method of Chomczynski and Sacchi [18] and mRNA was enriched by chromatography using oligo dT cellulose [19]. RNA was denatured by heating in 50% formamide and size fractionated on a 1% agarose gel containing formaldehyde. Following electrophoresis, the RNA was transferred to a nylon membrane and immobilized by UV cross linking. The bound RNA was hybridized to GPR26, ψGPR57, and GPR58 DNA fragments that had been radiolabeled with α-[^{32}P]dCTP to a specific activity of $\geq 1\times10^8$ dpm/μg. Specific hybrids were selected by washing the filter in $0.1\times$SSC, 0.1% SDS and detected by exposing the membrane to X-ray film at −70°C in the presence of an image intensifying screen.

2.10. In situ hybridization

In situ hybridization was performed as described previously [20]. Briefly, male rats (Jackson Laboratories, Bar Harbor, ME, USA) were killed and brains removed within 30 s and frozen on crushed dry ice. Sections (14 μm thickness) were cut at −20°C on a Reichert-Jung cryostat and mounted onto microscope slides. Sections were fixed in 4% paraformaldehyde, washed with phosphate buffered saline (pH 7.4) before dehydration. Rat GPR26 DNA was radiolabeled by random priming in the presence of [^{35}S]dCTP (New England Nuclear, Boston, MA, USA). Brain sections were incubated in hybridization buffer and after 2 h, the labeled DNA was added (1×10^6 dpm/slice) and incubation continued for 16 h. The sections were washed and dehydrated. Slides were exposed to X-ray film (Dupont MRF-34) for 4–6 weeks at −70°C. For use as controls, adjacent sections were hybridized following treatment with RNase.

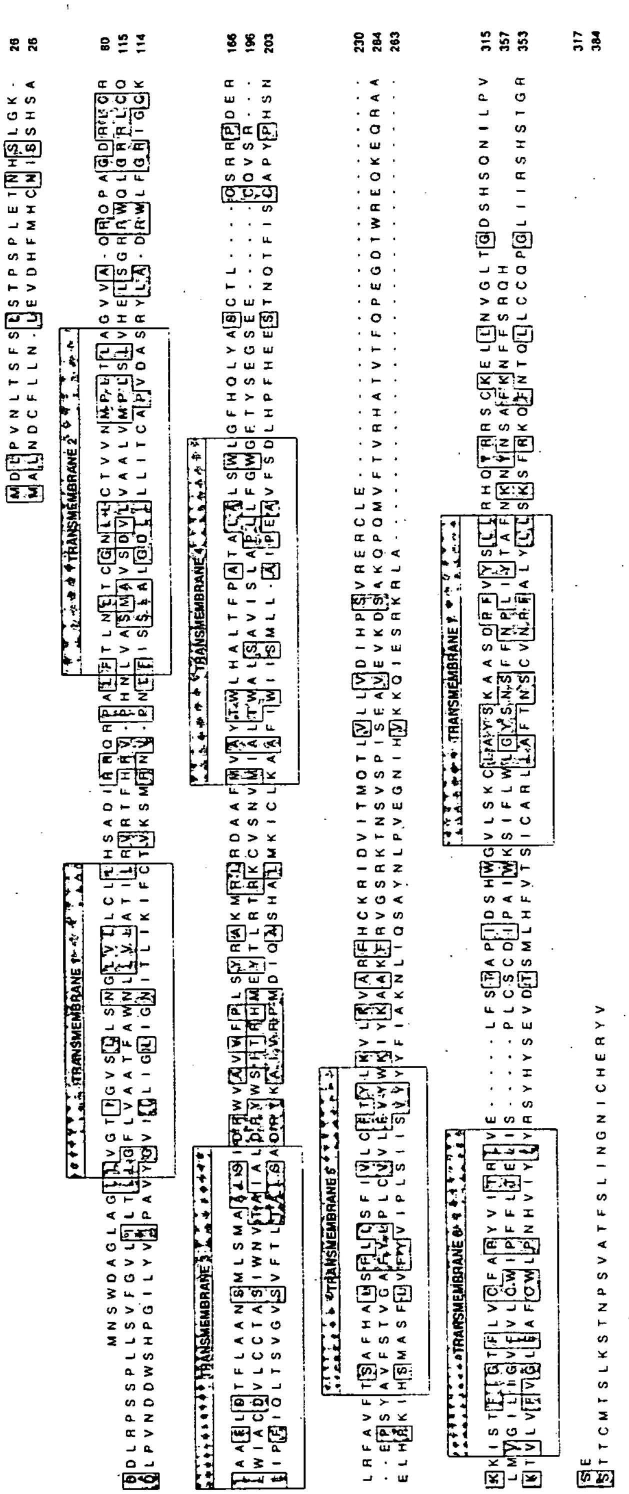
2.11. Chromosomal assignment of genes

Metaphase spread chromosomes derived from human lymphocytes were used for chromosomal assignment of the GPR26, ψGPR57 and GPR58 genes. Slides were prepared and fluorescence in situ hybridization (FISH) analysis done by probing with biotinylated phage clones encoding GPR26, ψGPR57 and GPR58 as previously described [21,22]. In addition, a Southern blot analysis of a human monochromosomal somatic cell hybrid panel (BIOS laboratories) was performed with a probe encoding ψGPR57 as per manufacturers instructions.

3. Results

3.1. Cloning of GPR26

A cDNA encoding GPR26 was first identified as a partial length human EST cDNA (cloneID_HIBB05). A fragment of the human GPR26 gene was obtained by screening a genomic DNA library and a 5.2 kb fragment isolated. Sequence comparison of the EST cDNA and the genomic DNA fragment showed that the sequences diverged in transmem-



brane region (TM) 6, indicating the presence of an intron in the human GPR26 gene. We used oligonucleotide primers partially encoding a single exon of the EST to amplify a fragment of the orthologous rat gene. The rat GPR26 fragment was used to screen a rat brain cDNA library, which resulted in the isolation of two phage clones encoding identical translational open reading frames. In keeping with the orphan GPCR nomenclature, this DNA was named GPR26.

The receptor encoded by the rat GPR26 cDNA was 317 amino acids long (Fig. 1), and most similar to a serotonin receptor (5-HT_{5A}) and the gastrin releasing hormone receptor (BB2). These proteins shared only 25% overall identity with the receptor encoded by GPR26, demonstrating a distant relationship to other Family A GPCRs. In the region of overlap (TM3 onward) with the partial human GPCR26 cDNA (280 amino acids), the rat and human GPR26 proteins were >95% identical. The GPR26 protein has a short amino terminus without the N-linked glycosylation motif, an unusual but not unique occurrence among GPCRs. A curious feature of the GPR26 encoded receptor was the occurrence of an arginine residue at position 251 (in TM6) and a lysine residue at position 270 (in TM7). This pair of cationic residues are found only in the purinergic P_2Y receptor cluster [23,24]. To determine whether GPR26 functions as a nucleotide receptor, we tested multiple 1321N1 cell populations following transfection with GPR26 for calcium mobilization in response to nucleoside di- and tri-phosphates. All of these assays proved negative, as did parallel assays in *Xenopus laevis* oocytes (data not shown).

3.2. Cloning of ψGPR57 and GPR58

Human genomic DNA was PCR amplified using primers based upon a patent (#EP 0859055-A/1) gene sequence HNHCI32. We obtained a gene very similar to HNHCI32 with the exception of two single

←
Fig. 1. Comparison of receptors encoded by rat GPR26, human 5-HT_{5A} and human BB2. The regions comprising the seven TMs are shown boxed. Residues identical between GPCRs are boxed and shaded. GPR26 sequence data has been deposited with GenBank (Accession # AF208288).

```
                                                5'- CTCATCCTCCTGGAAAGAAATTTCAAGGGATAAAGCACC
1                                                                                      98
ATG GAT CTA ACT TAT ATT CCC GAA GAC CTA TCC AGT TGT CCA AAA TTT GTA AAT AA* ATC
Met Asp Leu Thr Tyr Ile Pro Glu Asp Leu Ser Ser Cys Pro Lys Phe Val Asn     Ile
20                                                                                    158
CTG TCC TCC CAC CAA CCG CTC TTT TCA TGT CCA GGT GAT AAT GTA TTC GGT TAT GAC TGG
Leu Ser Ser His Gln Pro Leu Phe Ser Cys Pro Gly Asp Asn Val Phe Gly Tyr Asp Trp
40          TM 1                                                                  217
AGC CAT GAT TAT *CA CTA TTt GGA AAC TTG GTT ATA ATG GTT TCC ATA TCG CAT TTC AAA
Ser His Asp Tyr     Leu Phe Gly Asn Leu Val Ile Met Val Ser Ile Ser His Phe Lys
59                                                          TM 2                  277
CAG CTT CAC TCT CCC ACA AAC TTT CTG ATC CTC TCC ATG GCA ACC ACG GAC TTT CTG CTG
Gln Leu His Ser Pro Thr Asn Phe Leu Ile Leu Ser Met Ala Thr Thr Asp Phe Leu Leu
79                                                                                337
GGT TTT GTC ATT ATG CCA TAC AGC ATA ATG CGA TCA GTG GAG AGT TGC TGG TAC TTT GGG
Gly Phe Val Ile Met Pro Tyr Ser Ile Met Arg Ser Val Glu Ser Cys Trp Tyr Phe Gly
99                                                      TM 3                      397
GAT GGC TTT TGT AAA TTC CAC ACA AGC TTT GAC ATG ATG CTC AGA CTG ACC TCC ATT TTC
Asp Gly Phe Cys Lys Phe His Thr Ser Phe Asp Met Met Leu Arg Leu Thr Ser Ile Phe
119                                                                               457
CAC CTC TGT TCC ATT GCT ATT GAC CGA TTT TAT GCC GTG TGT TAC CCT TTA CAT TAC ACA
His Leu Cys Ser Ile Ala Ile Asp Arg Phe Tyr Ala Val Cys Tyr Pro Leu His Tyr Thr
139                                                     TM 4                      517
ACC AAA ATG ACG AAC TCC ACC ATA AAG CAA CTG CTG GCA TTT TGC TGG TCA GTT CCT GCT
Thr Lys Met Thr Asn Ser Thr Ile Lys Gln Leu Leu Ala Phe Cys Trp Ser Val Pro Ala
159                                                                               577
CTT TTT TCT TTT GGT TTA GTT CTA TCT GAG GCC GAT GTT TCC GGT ATG CAG AGC TAT AAG
Leu Phe Ser Phe Gly Leu Val Leu Ser Glu Ala Asp Val Ser Gly Met Gln Ser Tyr Lys
179                                                                               637
ATA CTT GTT GCT TGC TTC AAT TTC TGT GCC CTT ACT TTC AAC AAA TTC TGG GGG ACA ATA
Ile Leu Val Ala Cys Phe Asn Phe Cys Ala Leu Thr Phe Asn Lys Phe Trp Gly Thr Ile
199         TM 5                                                                  697
TTG TTC ACT ACA TGT TTC TTT ACC CCT GGC TCC ATC ATG GTT GGT ATT TAT GGC AAA ATC
Leu Phe Thr Thr Cys Phe Phe Thr Pro Gly Ser Ile Met Val Gly Ile Tyr Gly Lys Ile
219                                                                               757
TTT ATC GTT TCC AAA CAG CAT GCT CGA GTC ATC AGC CAT GTG CCT GAA AAC ACA AAG GGG
Phe Ile Val Ser Lys Gln His Ala Arg Val Ile Ser His Val Pro Glu Asn Thr Lys Gly
239                                                                               817
GCA GTG AAA AAA CAC CTA TCC AAG AAA AAG GAC AGG AAA GCA GCG AAG ACA CTG GGT ATA
Ala Val Lys Lys His Leu Ser Lys Lys Lys Asp Arg Lys Ala Ala Lys Thr Leu Gly Ile
259                     TM 6                                                      877
GTA ATG GGG GTG TTT CTG GCT TGC TGG TTG CCT TGT TTT CTT GCT GTT CTG ATT GAC CCA
Val Met Gly Val Phe Leu Ala Cys Trp Leu Pro Cys Phe Leu Ala Val Leu Ile Asp Pro
279                                                                               937
TAC CTA GAC TAC TCC ACT CCC ATA CTA ATA TTG GAT CTT TTA GTG TGG CTC CGG TAC TTC
Tyr Leu Asp Tyr Ser Thr Pro Ile Leu Ile Leu Asp Leu Leu Val Trp Leu Arg Tyr Phe
299                     TM 7                                                      997
AAC TCT ACT TGC AAC CCT CTT ATT CAT GGC TTT TTT AAT CCA TGG TTT CAG AAA GCA TTC
Asn Ser Thr Cys Asn Pro Leu Ile His Gly Phe Phe Asn Pro Trp Phe Gln Lys Ala Phe
319                                                                              1057
AAG TAC ATA GTG TCA GGA AAt ATA TTT AGC TCC CAT TCA GAA ACT GCA AAT TTG TTT CCT
Lys Tyr Ile Val Ser Gly Asn Ile Phe Ser Ser His Ser Glu Thr Ala Asn Leu Phe Pro
339                                                                              1114
GAA GCA CAT TAATAAAAAGCTGTTGCAAAAGTGAATAGAATATTGCAAATGAGATTG -3'
Glu Ala His
```

Fig. 2. Sequence of ψGPR57. Differences and deletions from GPR57 nucleotide sequence are indicated in lower case bold and by a '*' respectively. Without the frame shifts, the predicted TMs are shaded and labeled. Amino acids are numbered on the left and nucleotides on the right. Sequence data have been deposited with GenBank for ψGPR57 (Accession # AF112462).

base-pair substitutions and two single base-pair deletions. In order to search for a gene identical to the patent sequence containing an ORF encoding a complete GPCR, this DNA was used to screen a human genomic library. Two phages were identified and amplified by PCR with HNHC132 TM3 and TM7-encoding primers. This phage was PCR amplified with the HNHC132 5′ and 3′ UTR-encoding primers and a sequence analysis revealed both the amplified genomic DNA and genomic library gene products to be identical and both encoded a pseudogene, ψGPR57 (Fig. 2), sharing a 99.6% bp identity with HNHC132 (which was renamed GPR57).

We obtained a rabbit sequence as revealed in a patent (# JP 1997051795-A/1, GenBank Accession # E12664) which partially encoded a novel GPCR. This sequence was used in a BLAST [25] search of the GenBank GSS database to reveal human genomic clone 2005D7 (GenBank Accession # B52458) which partially encoded a receptor from the third intracellular loop to the stop codon with 92% identity to the rabbit sequence. Human genomic DNA was PCR amplified using a primer based on the TM6 encoded by clone 2005D7 and a degenerate primer designed from the sequence encoding TM3 of PNR, 5-HT_4 pseudogene, and the rabbit E12664 sequence. The PCR product (~400 bp) revealed an 89% identity to the patented rabbit sequence, confirming a human ortholog. This DNA was used to screen a human genomic library, revealing a phage encoding a novel GPCR, which we named GPR58. The sequence obtained was from the first extracellular loop to the stop codon, indicating the presence of an intron at the TM2/first extracellular loop junction. During the course of our work, a patent was released (# JP 1997238686-A/1, GenBank Accession # E13892) which included a gene named phBL5 encoding the human GPR58 ORF. To isolate the GPR58 ORF, two sets of human GPR58 primers were used to amplify the two exons flanking the TM2/first intracellular loop junction intron. The amplified fragments were joined in one round of PCR by the extension of primers whose sequences overlapped the two fragments and the ORF was amplified in a second PCR round with GPR58 5′ and 3′ UTR-specific primers.

GPR57 and GPR58 encoded proteins of 343 and 306 amino acids respectively. The sequence of the GPR58 ORF varied only by two thymine to cytosine substitutions compared with the phBL5 patent sequence, resulting in a translation of Ala^{133} in place of Val and a silent nucleotide variation encoding Tyr^{184}. Using GPR57 and GPR58 nucleotide and encoded protein sequences in BLAST searches, the highest identities were observed with the PNR and serotonin 5-HT_4 receptors and a reported 5-HT_4 pseudogene (Fig. 3). The GPR57 encoded receptor shared identities with the GPR58 (59%), PNR (37%), the 5-HT_4 (30%) receptors and a 5-HT_4 pseudogene (35%). The GPR58 encoded receptor shared identities with the PNR (42%), and 5-HT_4 (34%) receptors and the 5-HT_4 pseudogene (49%). Conserved residues and consensus sequences of Family A GPCRs present in the GPR57 and GPR58 receptors included an asparagine in TM1, an aspartate in TM2, prolines in TMs 4–7, cysteines in the first and second extracellular loops, and PKC consensus sequences in the second and third intracellular loops.

3.3. Expression analysis

To determine the expression patterns of GPR26, we performed northern blots of rat tissue. As shown in Fig. 4, GPR26 RNA was detected as a single band migrating at about 9 kb in extracts of several brain regions including striatum, pons, cerebellum and cortex. We failed to detect GPR26 mRNA in numerous peripheral tissue extracts excepting testis, where a 3 kb GPR26 RNA transcript was detected (not shown). To examine GPR26 expression in rat brain at higher resolution, we performed in situ hybridization. GPR26 RNA was detected in cortical structures including the anterior cingulate area, posterior cingulate and the frontoparietal, somatosensory and piriform cortices. The distribution of signal among the layers of cortex was not uniform, being mostly concentrated in layers I–III. GPR26 RNA was prominent also in the olfactory tubercle, the islands of Calleja, ventromedial and posterior nuclei of the hypothalamus, the medial septal nucleus, nucleus of the diagonal band and the ventral tegmental area. GPR26 RNA was localized also to hippocampal structures, with signals strongest over the CA2 and CA3 regions of Ammon's horn and less so over the dentate gyrus. The caudate putamen was labeled only in its most caudal portion, with a decreasing

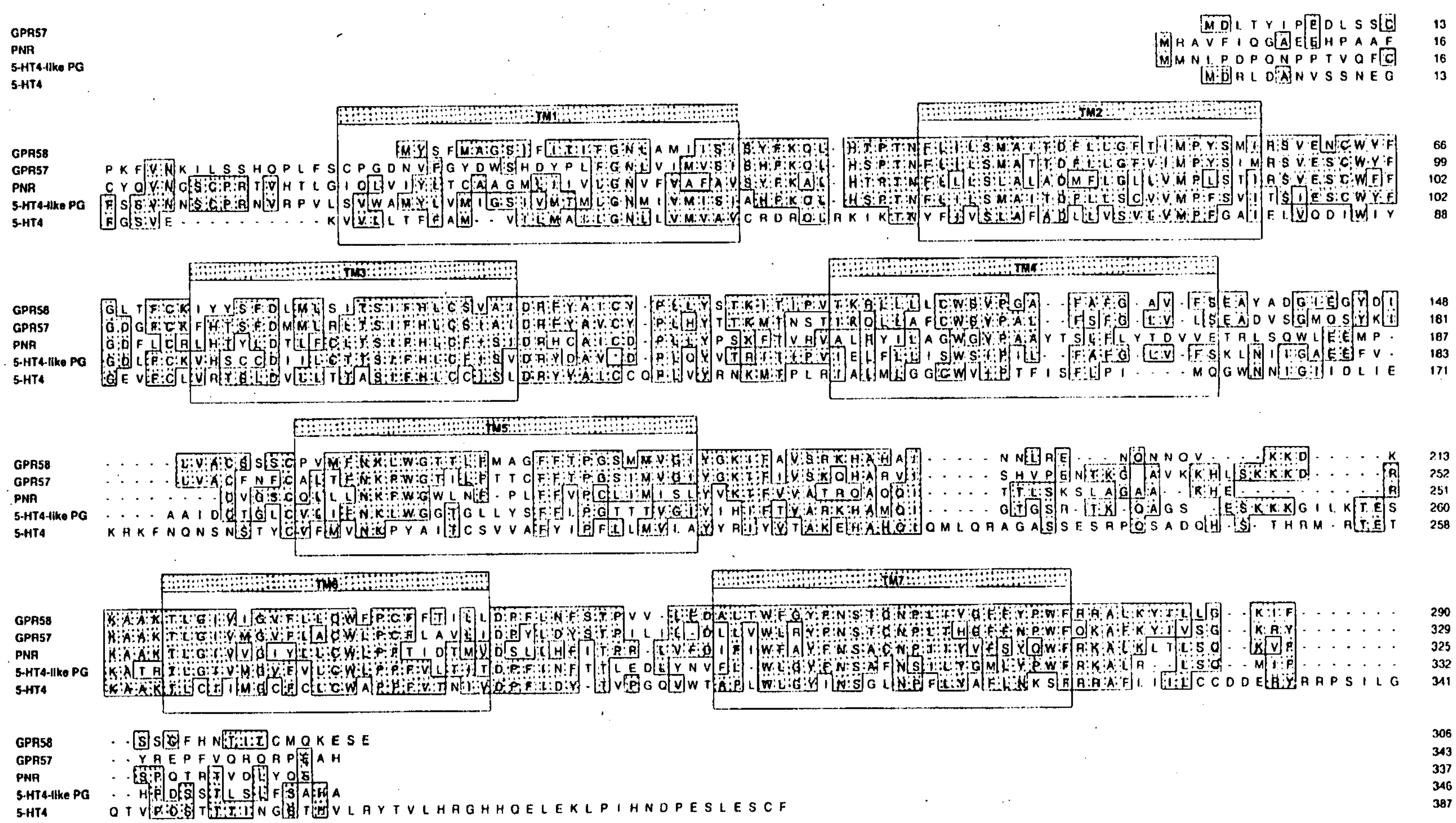


Fig. 3. Comparison of receptors encoded by GPR57, GPR58, PNR, 5-HT$_4$ and a 5-HT$_4$ pseudogene. The regions comprising the seven TMs are shown boxed. Residues identical between GPCRs are boxed and shaded. The in-frame stop codon in the 5-HT$_4$ pseudogene is indicated by a '*'. Sequence data have been deposited with GenBank for GPR57 (Accession # AF112461) and GPR58 (Accession # AF112460).

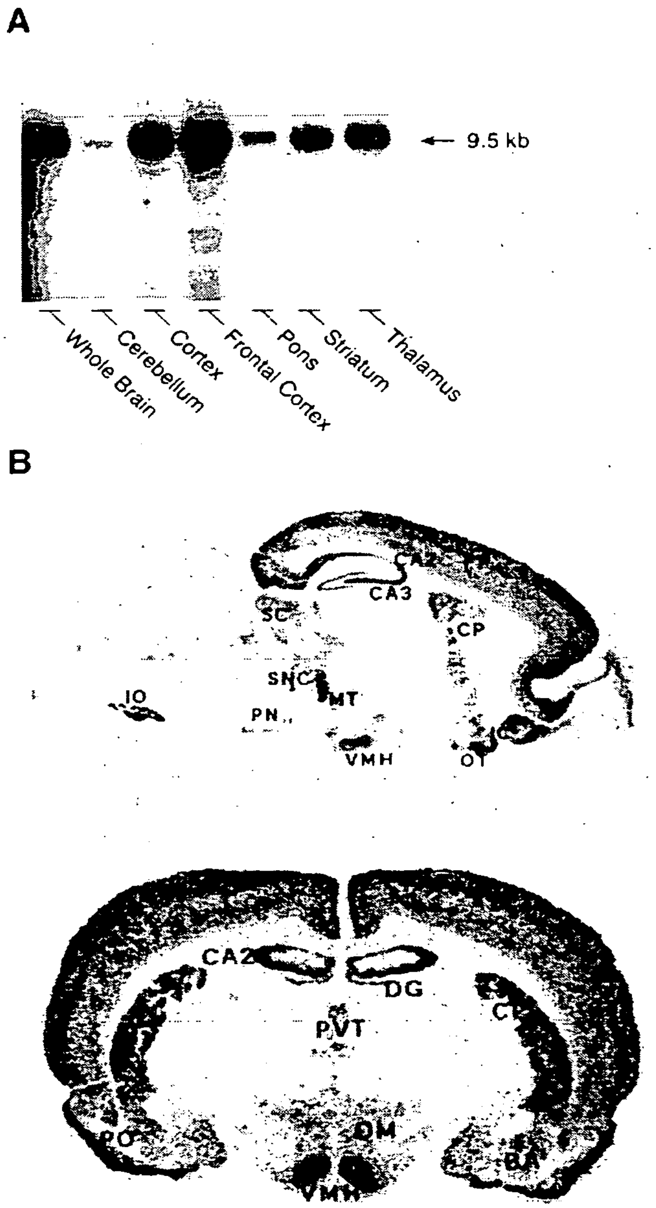


Fig. 4. Localization of rat brain GPR26 RNA. A: Northern blot of rat brain extracts. Five μg of poly (A^{+}) RNA was applied to each lane. Size estimation is from comparison with migration of RNA standards (not shown). B: In situ hybridization of sulfur-35 labeled GPR26 DNA. Upper panel: Sagittal section taken at the midline. Lower panel: Coronal section at the level of the hippocampus. Abbreviations: BA, basomedial amygdaloid nucleus; CP, caudate putamen; DG, dentate gyrus; DM, dorsomedial hypothalamic nucleus; F, frontal cortex; FP, frontal parietal cortex; IC, islands of Calleja; IO, inferior olivary nucleus; MT, medial terminal nucleus of the accessory optic tract; OT, olfactory tubercle; PN, pontine nucleus; PO, primary olfactory cortex; PVT, paraventricular thalamic nucleus; SC, superior colliculus; SNC, substantia nigra pars compacta; VMH, ventromedial hypothalamic nucleus.

gradient of signal from the dorsal to ventral aspect. Finally, a strong signal was associated with a single pontine structure, the inferior olivary nucleus.

Northern analysis was also performed for GPR57 and GPR58 on human tissue. Since the GPR57 and ψGPR57 sequences shared 99.6% identity, the ψGPR57 probe would also detect GPR57 mRNA transcripts. For GPR57, revealed in the patent (# EP 0859055-A/1) to have been cloned from human hippocampus cDNA, no visible transcripts were detected in the pons, thalamus, globus pallidus, caudate, putamen or cerebellum. For GPR58 (revealed in patent # JP 1997051795-A/1 cloned from rabbit smooth muscle cDNA and in patent # JP 1997238686-A/1 from human cerebellum cDNA) no visible transcripts were detected in the pons, thalamus, hypothalamus, hippocampus, caudate, putamen, frontal cortex, basal forebrain, midbrain or liver.

3.4. *Attempted pharmacological characterization*

In order to test candidate ligands for GPR26, we introduced its cDNA into HEK293 cells by DNA-mediated transfection. With repeated experiments we observed a low efficiency of transfection (as measured by the number of colonies/dish), a slow growth rate of surviving clonal populations and derange-

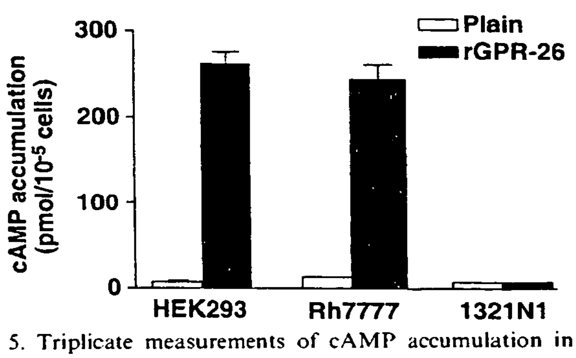
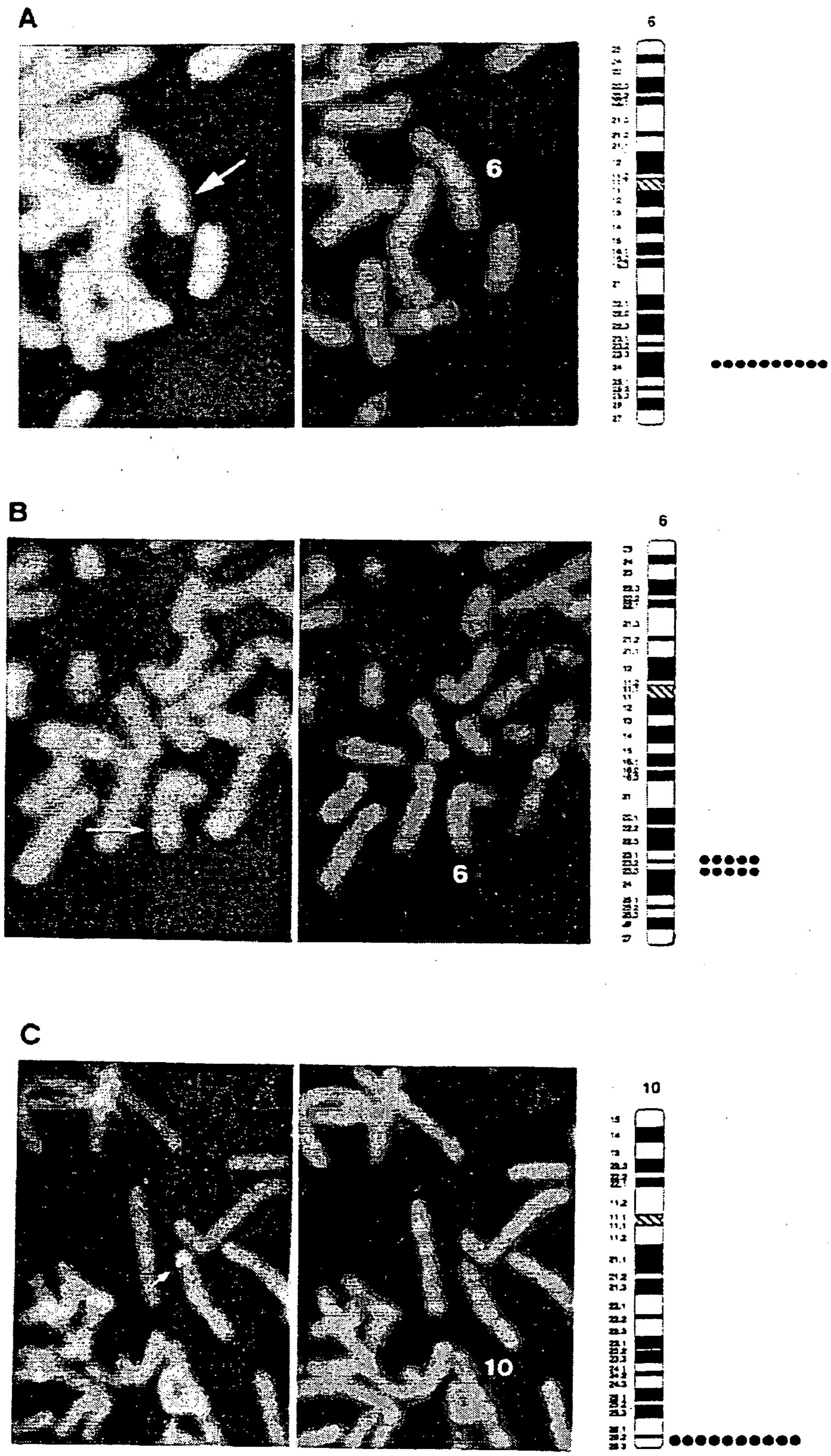


Fig. 5. Triplicate measurements of cAMP accumulation in the presence of IBMX in clonal cell lines transfected with rat GPR26 cDNA or mock transfected cells.


A
6
6
B
6
6
C
10
10

ments of normal cell shape. We hypothesized that these phenotypes might be the consequence of high basal cAMP levels in the transfected cells. As shown in Fig. 5 for one clonal population of HEK293 cells, cAMP levels were elevated as compared to mock transfected cultures. A similar situation was observed with six different clones of transfected Rh7777 cells (one is shown in Fig. 5). However, this phenomenon was not observed in any of eight individual 1321N1 cell clones transfected with GPR26 cDNA.

COS-7 cells were transfected with constructs encoding PNR and GPR58 and the membranes harvested for ligand binding assays. For the receptor encoded by PNR, several tritiated serotonin ligands, including spiperone, LSD, 5-HT, 5CT, 8-OH-DPAT and ketanserin were tested for binding to the expressed receptor. A selective tritiated 5-HT_4 receptor ligand, GR113808, was also used in an attempt to label the expressed receptor. No specific binding to the PNR receptor was detected with any ligands tested. For the receptor encoded by GPR58, $[^3H]$5-HT was tested for binding in competition experiments with three compounds: methiothepin (a 5-HT_1 antagonist), mianserin (a non-selective 5-HT antagonist) and SDZ-205,557 HCl (a 5-HT_4 antagonist). In each experiment, no specific binding of serotonin to the GPR58 receptor was detected.

3.5. *Chromosomal analysis*

A Southern blot of a human monochromosomal somatic cell hybrid panel with a probe encoding ψGPR57 was performed. Given the 99.6% sequence identity between ψGPR57 and GPR57, we expected to see bands revealing the chromosomal localization of both ψGPR57 and GPR57. A single band was detected (data not shown) suggesting that both ψGPR57 and GPR57 localized to chromosome 6.

FISH analysis of human metaphase spread chromosomes was used to identify the chromosomal localization of GPR26, ψGPR57 and GPR58. The phage clones were biotinylated and used for FISH mapping. The detailed positions were determined based on the summary from 20 photographs of human chromosome 10, region q26.2–q26.3 for GPR26, chromosome 6 region q23–q24 for ψGPR57 and chromosome 6 region q24 for GPR58 (Fig. 6). Previous reports have localized the receptor PNR at 6q23 [10] and the 5-HT_4 pseudogene at 6q22.1 [11]. Together, ψGPR57, GPR58, PNR and the 5-HT_4 pseudogene appear to compose a family of oGPCR genes that localize between q22 and q24 on chromosome 6.

4. Discussion

Based on the presence of two cationic residues, found similar to those found only in the P_2Y receptors, we tested nucleoside di- and tri-phosphates for binding with the receptor encoded by GPR26. This cationic amino acid pair has been suggested from modeling and mutagenesis studies to participate in the recognition of the α phosphate of ATP by the P_2Y_1 receptor [23,24]. However, assays with 1321N1 and *Xenopus laevis* oocyte cell lines transfected with the GPR26 receptor proved negative. The massive accumulation of cAMP in HEK293 and Rh7777 cells following GPR26 DNA transfection, which occurred in the absence of exogenous ligand, led us to suspect that GPR26 might exhibit high constitutive activity. However, cAMP levels were not found to be elevated in 1321N1 cells transfected with GPR26 DNA (eight clones were tested). Thus we predict that the ligand for the GPR26 receptor is an autocrine mediator released by HEK293 and Rh7777 cells, but not by the 1321N1 cells.

The ψGPR57 sequence varied from GPR57 by two single nucleotide deletions and two nucleotide substitutions, maintaining an overall nucleotide identity of 99.6% (Fig. 2). The two deletions resulted in two frame shifts relative to GPR57 in the regions of the gene corresponding to the extracellular N-terminal segment and TM1 respectively, the first leading to

←

Fig. 6. FISH analysis of ψGPR57 (A), GPR58 (B) and GPR26 (C) showing results of metaphase spread chromosomes probed with phage clones encoding ψGPR57, GPR58 or GPR26 and an ideogram summarizing the results of both FISH analyses. Each dot represents the location of a fluorescent signal on the chromosome using phage containing the ψGPR57, GPR58 or GPR26 clone as a probe.

a stop codon in TM3 and an ORF of only 309 bp encoding a 103 amino acid protein. We have previously reported other GPCR pseudogenes ψDRD5-1, ψDRD5-2 [26], ψ5-HTR$_{1D}$ [27], ψGPR32 and ψGPR33 [28], and ψGPR53 [29].

The GPR58 receptor does not appear to encode an extracellular N-terminal segment. While other GPCRs with very short extracellular N-terminal segments have been identified (e.g. GPR26, the adenosine A1 receptor [30], A2b receptor [31] and histamine H2 receptor [32]), the ORF of GPR58 appeared to start within the first TM with the identified initiation methionine conformed to an adequate Kozak sequence [33].

Both GPR57 and GPR58 displayed residues important for ligand binding comparable to those in biogenic amine-binding receptors. Specifically, an aspartate in TM3, threonine in TM5 and phenylalanine in TM6 shown to be important for ligand binding and stability in biogenic amine-binding receptors [34] were conserved in both the GPR57 and GPR58 receptors. While two members of this subfamily, PNR and GPR58, did not bind serotonin; based on sequence identities and the conservation of several important residues with amine-binding GPCRs, we predict these receptors will bind an endogenous amine-like ligand, perhaps of a type yet to be discovered.

Acknowledgements

This research was funded by DHHS USPHS NIH R01 GM52722 (K.R.L.), the Medical Research Council of Canada (B.F.O. and S.R.G.), the National Institute on Drug Abuse (B.F.O. and S.R.G.) and by a PMAC/MRC grant from Merck Frosst Research Laboratories, Inc. (B.F.O. and S.R.G.).

References

[1] A. Marchese, S.R. George, L.F. Kolakowski Jr., K.R. Lynch, B.F. O'Dowd, Novel G protein-coupled receptors and their endogenous ligands: expanding the boundaries of physiology and pharmacology, Trends Pharmacol. Sci. 20 (1999) 370–375.

[2] J.-C. Meunier, C. Mollereau, L. Toll, C. Suaudeau, C. Moisand, P. Alvinerie, J.-L. Butour, J.-C. Guillemot, P. Ferrara, B. Monsarrat et al., Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor, Nature 377 (1995) 532–535.

[3] R.K. Reinscheid, H.-P. Nothacker, A. Bourson, A. Ardati, R.A. Henningsen, J.R. Bunzow, D.K. Grandy, H. Langen, F.J. Monsma Jr., O. Civelli, Orphanin FQ: A neuropeptide that activates an opioidlike G protein-coupled receptor, Science 270 (1995) 792–794.

[4] T. Sakurai, A. Amemiya, M. Ishii, I. Matsuzaki, R.M. Chemelli, H. Tanaka, S.C. Williams, J.A. Richardson, G.P. Kozlowski, S. Wilson et al., Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behaviour, Cell 92 (1998) 573–585.

[5] S. Hinuma, Y. Habata, R. Fujii, Y. Kawamata, M. Hosoya, S. Fukusumi, C. Kitada, Y. Masuo, T. Asano, H. Matsumoto et al., A prolactin-releasing peptide in the brain, Nature 393 (1998) 272–276.

[6] K. Tatemoto, M. Hosoya, Y. Habata, R. Fujii, T. Kakegawa, M.-X. Zou, Y. Kawamata, S. Fukusumi, S. Hinuma, C. Kitada et al., Isolation and characterization of a novel endogenous peptide ligand for the human APJ receptor, Biochem. Biophys. Res. Commun. 251 (1998) 471–476.

[7] O. Civelli, Functional genomics: the search for novel neurotransmitters and neuropeptides, FEBS Lett. 430 (1998) 55–58.

[8] J.M. Stadel, S. Wilson, D.J. Bergsma, Orphan G protein-coupled receptors: a neglected opportunity for pioneer drug discovery, Trends Pharmacol. Sci. 18 (1997) 430–437.

[9] K.R. Lynch, G protein-coupled receptor informatics and the orphan problem, in: K.R. Lynch (Ed.), Identification and Expression of G Protein-Coupled Receptors, Wiley-Liss, New York, 1998, pp. 54–72.

[10] Z. Zeng, P. Fan, E. Rand, H. Kyaw, K. Su, V. Madike, K.C. Carter, Y. Li, Cloning of a putative human neurotransmitter receptor expressed in skeletal muscle and brain, Biochem. Biophys. Res. Commun. 242 (1998) 575–578.

[11] I.S.C. Liu, I. Kusumi, C. Ulpian, T. Tallerico, P. Seeman, A serotonin-4 receptor-like pseudogene in humans, Brain Res. Mol. Brain Res. 53 (1998) 98–103.

[12] M. Wigler, S. Silverstein, L.S. Lee, A. Pellicer, Y.C. Cheng, R. Axel, Transfer of purified Herpes virus thymidine kinase gene into cultured mouse cells, Cell 11 (1977) 223–232.

[13] A. Marchese, J.M. Docherty, T. Nguyen, M. Heiber, R. Cheng, H.H. Heng, L.C. Tsui, X. Shi, S.R. George, B.F. O'Dowd, Cloning of human genes encoding novel G protein-coupled receptors, Genomics 23 (1994) 609–618.

[14] S.B. Hooks, S.P. Ragan, D.W. Hopper, C.W. Honemann, M.E. Durieux, T.L. Macdonald, K.R. Lynch, Characterization of a receptor subtype-selective lysophosphatidic acid mimetic, Mol. Pharmacol. 53 (1998) 188–194.

[15] K.R. Lynch, D.W. Hopper, S.J. Carlisle, J.G. Catalano, M. Zhang, T.L. Macdonald, Structure-activity relationships in lysophosphatidic acid: the 2-hydroxyl moiety, Mol. Pharmacol. 52 (1997) 75–81.

[16] I.S. Liu, P. Seeman, S. Sanyal, C. Ulpian, P.E. Rodgers-Johnson, G.R. Serjeant, H.H. Van Tol, Dopamine D4 re-

ceptor variant in Africans. D4valine194glycine. is insensitive to dopamine and clozapine: report of a homozygous individual. Am. J. Med. Genet. 61 (1996) 277–282.

[17] G.Y. Ng. S.R. George. R.L. Zastawny. M. Caron. M. Bouvier. M. Dennis. B.F. O'Dowd. Human serotonin1B receptor expression in Sf9 cells: phosphorylation. palmitoylation. and adenylyl cyclase inhibition. Biochemistry 32 (1993) 11727–11733.

[18] P. Chomczynski. N. Sacchi. Single-step methods of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162 (1988) 156–159.

[19] H. Aviv. P. Leder. Purification of biologically active globin mRNA by chromatography on oligothymidylic acid-cellulose. Proc. Natl. Acad. Sci. USA 69 (1972) 1408–1412.

[20] R.L. Zastawny. S.R. George. T. Nguyen. R. Cheng. J. Tsatsos. R. Briones-Urbina. B.F. O'Dowd. Cloning. characterization and distribution of a mu opioid receptor in rat brain. J. Neurochem. 62 (1994) 2099–2105.

[21] H.H. Heng. J. Squire. L.C. Tsui. High-resolution mapping of mammalian genes by in situ hybridization to free chromatin. Proc. Natl. Acad. Sci. USA 89 (1992) 9509–9513.

[22] H.H. Heng. L.C. Tsui. Modes of DAPI banding and simultaneous in situ hybridization. Chromosoma 102 (1993) 325–332.

[23] L. Erb. R. Garrad. Y. Wang. T. Quinn. J.T. Turner. G.A. Weisman. Site-directed mutagenesis of P2U purinoceptors. Positively charged amino acids in transmembrane helices 6 and 7 affect agonist potency and specificity. J. Biol. Chem. 270 (1995) 4185–4188.

[24] Q. Jiang. D. Guo. B.X. Lee. A.M. van Rhee. Y.C. Kim. R.A. Nicholas. J.B. Schachter. T.K. Harden. K.A. Jacobson. A mutation analysis of residues essential for ligand recognition at the human P2Y1 receptor. Mol. Pharmacol. 52 (1997) 499–507.

[25] S.F. Altschul. T.L. Madden. A.A. Schaffer. J. Zhang. Z. Zhang. W. Miller. D.J. Lipman. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25 (1997) 3389–3402.

[26] T. Nguyen. J. Bard. H. Jin. D. Taruscio. D.C. Ward. J.L. Kennedy. R. Weinshank. P. Seeman. B.F. O'Dowd. Human dopamine D5 receptor pseudogenes. Gene 109 (1991) 211–218.

[27] T. Nguyen. A. Marchese. J.L. Kennedy. A. Petronis. S.J. Peroutka. P.H. Wu. B.F. O'Dowd. An Alu sequence interrupts a human 5-hydroxytryptamine1D receptor pseudogene. Gene 124 (1993) 295–301.

[28] A. Marchese. T. Nguyen. P. Malik. S. Xu. R. Cheng. Z. Xie. H.H. Heng. S.R. George. L.F. Kolakowski Jr.. B.F. O'Dowd. Cloning genes encoding receptors related to chemoattractant receptors. Genomics 50 (1998) 281–286.

[29] M. Sawzdargo. T. Nguyen. D.K. Lee. K.R. Lynch. R. Cheng. H.H. Heng. S.R. George. B.F. O'Dowd. Identification and cloning of three novel human G protein-coupled receptor genes GPR52. PsiGPR53 and GPR55: GPR55 is extensively expressed in human brain. Brain Res. Mol. Brain Res. 64 (1999) 193–198.

[30] A. Townsend-Nicholson. J. Shine. Molecular cloning and characterisation of a human brain A1 adenosine receptor cDNA. Brain Res. Mol. Brain Res. 16 (1992) 365–370.

[31] K.D. Pierce. T.J. Furlong. L.A. Selbie. J. Shine. Molecular cloning and expression of an adenosine A2b receptor from human brain. Biochem. Biophys. Res. Commun. 187 (1992) 86–93.

[32] I. Gantz. G. Munzert. T. Tashiro. M. Schaffer. L. Wang. J. DelValle. T. Yamada. Molecular cloning of the human histamine H2 receptor. Biochem. Biophys. Res. Commun. 178 (1991) 1386–1392.

[33] M. Kozak. Interpreting cDNA sequences: some insights from studies on translation. Mamm. Genome 7 (1996) 563–574.

[34] C.D. Strader. T.M. Fong. M.R. Tota. D. Underwood. R.A. Dixon. Structure and function of G protein-coupled receptors. Annu. Rev. Biochem. 63 (1994) 101–132.

THIS PAGE BLANK (USPTO)